



Short communication

## A new atypical genotype mouse virulent strain of *Toxoplasma gondii* isolated from the heart of a wild caught puma (*Felis concolor*) from Durango, Mexico



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### ABSTRACT

Nothing is known of the genetic diversity of *Toxoplasma gondii* circulating in wildlife in Mexico. In the present study, a mouse virulent *T. gondii* strain was isolated from the heart of a wild puma (*Felis concolor*). The puma was found roaming in outskirt of Durango City, Mexico and tranquilized for moving to a zoo. The puma died during translocation and a necropsy examination was performed. The puma had an antibody titer for *T. gondii* of 200 by the modified agglutination test. Its heart and brain tissue were bioassayed into 2 outbred Swiss Webster (SW) and 1 gamma interferon gene knockout (KO) mouse. The KO mouse and the 2 SW mice that became infected after inoculation with homogenate of puma heart died of acute toxoplasmosis 12, 19 and 20 days p.i. respectively and tachyzoites were found in lungs of all 3 mice. None of the 4 SW and 1 KO mouse inoculated with digest of the puma brain became infected with *T. gondii*. Tachyzoites from the lungs of mice were propagated in cell cultures. Tachyzoites from cell culture were inoculated into 5 SW; the mice died or had to be killed 14 days p.i. and a cat fed tissues of these mice shed *T. gondii* oocysts. Results of mortality and infectivity of tachyzoites and oocysts in SW mice indicated that the puma *T. gondii* strain (designated TgPumaMe1) was virulent for outbred mice. DNA isolated from culture-derived tachyzoites was characterized using 11 PCR-RFLP markers (SAG1, 5'- and 3'-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) revealed a new genotype (ToxoDB PCR-RFLP #222). Isolation of atypical genotype *T. gondii* from wild puma indicates that mouse virulent strains are circulating in wildlife in Mexico.

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### 1. Introduction

The protozoan parasite *Toxoplasma gondii* infects virtually all warm-blooded animals, including birds, humans, livestock, and marine mammals (Dubey, 2010). Humans become infected postnatally by ingesting tissue cysts from

undercooked meat, or by consuming food or drink contaminated with oocysts. However, only a small percentage of exposed adult humans or other animals develop clinical signs of disease. It is unknown whether the severity of toxoplasmosis in immunocompetent hosts is due to the parasite strain, host variability, or to other factors. Recently, attention has been focused on the genetic variability among *T. gondii* isolates from apparently healthy and sick hosts (Grigg and Sundar, 2009). Severe cases of toxoplasmosis have been reported in immunocompetent

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patients in association with atypical *T. gondii* genotypes in certain countries (Ajzenberg et al., 2004; Demar et al., 2007; Elbez-Rubinstein et al., 2009; Grigg and Sundar, 2009).

Historically, *T. gondii* was considered to be clonal with low genetic diversity and grouped into three types I, II, III (Howe and Sibley, 1995; Su et al., 2012). However, recent studies have revealed a greater genetic diversity of *T. gondii*, particularly isolates from domestic animals in Brazil and wildlife in the USA (Dubey et al., 2011; Khan et al., 2011; Dubey et al., 2012; Su et al., 2012).

Nothing is known of clinical toxoplasmosis in humans and its association with genetic diversity in Mexico. Here we report first isolation and genetic characterization of *T. gondii* from a wild puma (*Felis concolor*) in Mexico.

## 2. Materials and methods

### 2.1. Naturally infected puma

On November 11, 2012 a female 5-year-old puma (*Felis concolor*) was found roaming in the external northwest boundary of Durango City, Mexico in a residential area near the Sierra Madre Occidental mountain, the Sahuatoba and the city zoo ( $24^{\circ}01' N$ ,  $104^{\circ}40' W$ ). The puma was tranquilized by a dart by the municipal authorities because of concern of human safety. The puma was transported to the zoo but, died soon after arrival. A necropsy examination was performed. After viewing the local news on TV (<http://laguna.milenio.com/cdb/doc/noticias2011/9fddaf82c2070764965d3420fecabb2d>) in Durango City, one of us (Alvarado-Esquivel) contacted the zoo for possible collection of samples for the present study. By the time we contacted the zoo, tissues had been stored in a freezer; tissues were not fixed for histopathological examination. Tissues were retrieved from the freezer (tissues had not become solid and had been there only for few hours). Heart, brain, and feces were collected for the present study and transported by air to the Animal Parasitic Diseases Laboratory (APDL), United States Department of Agriculture in Beltsville, Maryland for *T. gondii* examination. Samples were received at APDL 4 days later.

### 2.2. Serology

Sera from animals were tested for antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Sera were diluted two-fold serially from 1:25 to 1:200.

### 2.3. Bioassay in mice

The heart and brain were homogenized individually, digested in acidic pepsin, washed, and aliquots of homogenates were inoculated subcutaneously into 4 outbred Swiss Webster (SW) mice and 1 gamma interferon gene knockout (KO) mouse (Dubey, 2010). Tissue imprints of lungs and brains of inoculated mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 45 post-inoculation (p.i.) and a 1:25 dilution of serum was tested for *T. gondii* antibodies

by MAT. Mice were killed 46 days p.i. and brains of all mice were examined for tissue cysts as described (Dubey, 2010). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues. Oocysts of the puma isolate were obtained by feeding infected mouse tissues as described (Dubey, 2010).

### 2.4. Pathogenicity of oocysts and tachyzoites of the puma *T. gondii* strain in SW mice

Pathogenicity of oocysts of the *T. gondii* isolate derived from the puma was done in SW mice (Table 1). Oocysts were sporulated in 2% sulfuric acid for a week on a shaker at room temperature, washed, counted, and diluted serially 10-fold from  $10^{-1}$  to  $10^{-7}$  to reach an end point of  $\geq 1$  oocyst. Mortality was recorded, and after two months mice were tested for *T. gondii* infection (Dubey, 2010). Mice were considered uninfected when antibodies to *T. gondii* were not demonstrable in their sera and parasites were not found.

For pathogenicity of tachyzoites, lungs of a SW mouse euthanized 14 days p.i. was homogenized in saline with pestle and mortar, and filtered through gauze. The suspension was passed through a 5- $\mu m$  micropore filter to remove any intact host cells and to ensure extracellular tachyzoites. The filtrate was diluted serially 10-fold to reach an end point of  $\geq 1$  tachyzoite (Table 1).

### 2.5. In vitro cultivation

Mouse tissues infected with *T. gondii* were seeded onto CV1 cell culture flasks and tachyzoites were harvested from the medium (Dubey, 2010).

### 2.6. Genetic characterization

*Toxoplasma gondii* DNA was extracted from cell-cultured tachyzoites and strain typing was performed using the genetic markers SAG1, 5'- and 3'-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico as described previously (Su et al., 2010). Appropriate controls were included (Table 2).

**Table 1**

Pathogenicity of oocysts and tachyzoites derived from *T. gondii* isolate from the puma to Swiss Webster mice.<sup>a</sup>

Dose <sup>b</sup>	TgPumaMe1 (Cat 49)	
	Oocysts	Tachyzoites
10	4 (17, 17, 18, 20) <sup>c</sup>	5 (13, 13, 21, 21, 22)
1	1 (17)	5 (13, 16, 28, 28, 28)
<1	0	0

<sup>a</sup> Five mice per group. Oocysts were inoculated orally, tachyzoites were inoculated subcutaneously.

<sup>b</sup> No. of mice infected with *T. gondii* of 5 mice inoculated. Day of death of each mouse is in parenthesis. Mice not shown in parenthesis did not die.

<sup>c</sup> Based on estimation that the last infective dilution has 1 infective organism.

**Table 2**Genetic characterization of *T. gondii* isolate from the puma from Mexico.

Strain ID	Genotypes (ToxoDB PCR-RFLP genotypes)	Genetic markers (Su et al., 2010)										
		SAG1	(5' + 3') SAG2 <sup>a</sup>	alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
GT1	Type I (#10)	I	I	I	I	I	I	I	I	I	I	I
PTG	Type II (#1)	II	II	II	II	II	II	II	II	II	II	II
CTG	Type III (#2)	II or III	III	III	III	III	III	III	III	III	III	III
MAS	Atypical (#17)	u-1 <sup>b</sup>	I	II	III	III	III	u-1	I	I	III	I
TgCgCa1	Atypical (#66)	I	II	II	III	II	II	II	u-1	I	u-2	I
TgCtBr5	Atypical (#19)	I	III	III	III	III	I	I	I	I	u-1	I
TgCtBr64	Atypical (#111)	I	I	u-1	III	III	III	u-1	I	III	III	I
TgRsCr1	Atypical (#52)	u-1	I	II	III	I	III	u-2	I	I	III	I
<b>Present study</b>												
TgPumaMe1	Atypical (#222)	I	II	II	II	II	II	II	u-1	I	II	I

<sup>a</sup> The markers (5' + 3') SAG2 and alt.SAG2 target at different DNA fragments in SAG2 gene.<sup>b</sup> The u-1 and u-2 are new alleles different from the conventional types I, II or III alleles.

## 2.7. Ethics

All investigations reported here were approved by the institutional animal use protocol committee of the United States Department of Agriculture.

## 3. Results

The puma had a *T. gondii* antibody titer of 200. Viable *T. gondii* was isolated from the heart but, not from the brain of puma by bioassay in mice. Of the 4 SW mice inoculated with heart homogenate of puma, 2 mice died of bacterial infection day 3 p.i. and they were discarded. The other 2 SW mice died of acute toxoplasmosis 19, 20 days p.i. The KO mouse died day 12 p.i. Tachyzoites were found in smears made from lungs of all 3 infected mice.

Tachyzoites were propagated in cell culture. Tachyzoites from cell culture were inoculated into 5 SW mice; all 5 mice died or had to be euthanized day 14 p.i.; the cat fed acutely infected mice shed *T. gondii* oocysts.

The 4 SW mice and 1 KO mouse inoculated with brain homogenate of puma remained asymptomatic, and neither *T. gondii* parasites nor *T. gondii* antibodies were found in these 5 mice killed 53 days p.i.

*Toxoplasma* – like oocysts were not found by microscopic examination of fecal float of puma feces. The mice inoculated with aerated puma feces remained negative for *T. gondii*.

Genotyping of this *T. gondii* isolate revealed a new genotype, designated as ToxoDB PCR-RFLP genotype #222 (Table 2).

## 4. Discussion

Wild felids are important in the epidemiology of *T. gondii* because they prey on cervids and other animals in the wild and spread toxoplasmosis. The puma (also called Florida panther, mountain lion, and cougar) is a highly adaptable cat found in North and South America. It has a range of up to 80 km<sup>2</sup>, and feeds on a variety of mammals and birds, including livestock. Worldwide seroprevalence of *T. gondii* in *Felis concolor* were recently summarized (Jones and Dubey, 2010; Dubey, 2010). Kikuchi et al. (2004) found *T. gondii* antibodies in sera of 2 of 12 *F. concolor*

from Mexico. The number of puma in whole Mexico is unknown but, surveys in the municipality of Nacori Chico, Sonora, in the foothills of northern Sierra Madre Occidental (where this puma was caught) was estimated at 1.7/100 km<sup>2</sup> (Rosas-Rosas and Bender, 2012).

Nothing is known of clinical toxoplasmosis and genotyping of *T. gondii* from animals or humans in Mexico. Of the 13 *T. gondii* isolates from dogs (3), domestic cats (5), and chickens (5), from Mexico, 4 isolates from chickens were type III (ToxoDB PCR-RFLP genotype #2), and 9 were atypical (1 was #155, 3 were #74, 4 were #9, 1 was #73). All of these 13 isolates were avirulent for SW mice (Dubey et al., 2009). The ToxoDB genotype #73 was previously identified in sheep from the USA (Dubey et al., 2008a), the #74 was reported in sheep and white-tailed deer from the USA (Dubey et al., 2008a, 2008b), and the #9 was a dominant type in China (Chen et al., 2011).

In the present study, *T. gondii* was isolated from the heart muscle and not from the brain of the cougar. This is in keeping with the observations that *T. gondii* is more prevalent in muscles than the brains of livestock species and cats (Dubey, 2010).

Pathogenicity of *T. gondii* in outbred mice varies with the dose, route of inoculation, and the stage of the parasite inoculated. In general oocysts are more pathogenic than bradyzoites and tachyzoites and infections are more severe after oral inoculation. In the present case all three stages of *T. gondii* from the puma were virulent for outbred mice. The SW mice inoculated with puma heart digest died of acute toxoplasmosis. The puma was asymptomatic and its heart has been digested in pepsin, indicating that heart digest contained bradyzoites. SW mice inoculated with tachyzoites and oocysts also died of toxoplasmosis, irrespective of the dose.

Miller et al. (2008) tested 27 mountain lions from California for *T. gondii* infection. DNA was extracted from naturally infected animals and tested for *T. gondii* by multilocus PCR-DNA sequencing at the B1, SAG1, and GRA6 genes. In total, 5 animals were PCR positive; based on B1 gene sequencing, 2 samples were type X, 1 was type I, and 1 was type II (also GRA 6); *T. gondii* was not isolated in cell culture from any lion—personal communication to JPD from M.A. Miller, 6 March 2013.

Viable *T. gondii* was previously isolated from feces of 2 naturally infected Vancouver Island cougars (*Felis concolor vancouverensis*) from Canada (Aramini et al., 1998; Dubey et al., 2008c). Both of these isolates are atypical (ToxoDB PCR-RFLP genotype #66). The present isolate is first from tissues of a wild cougar and first isolate from a wild animal in Mexico.

## Conflict of interest

None.

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